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Dephosphorylation of photosystem II proteins and phosphorylation of CP29 in barley photosynthetic membranes as a response to water stress

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ABSTRACT

Kinetic studies of protein dephosphorylation in barley thylakoid membranes revealed accelerated dephosphorylation of photosystem II (PSII) proteins, and meanwhile rapidly induced phosphorylation of a light-harvesting complex (LHCII) b4, CP29 under water stress. Inhibition of dephosphorylation aggravates stress damages and hampers photosystem recovery after rewatering. This increased dephosphorylation is catalyzed by both intrinsic and extrinsic membrane protein phosphatase. Water stress did not cause any thylakoid destacking, and the lateral migration from granum membranes to stroma-exposed lamellae was only found to CP29, but not other PSII proteins. Activation of plastid proteases and release of TLP40, an inhibitor of the membrane phosphatases, were also enhanced during water stress. Phosphorylation of CP29 may facilitate disassociation of LHCII from PSII complex, disassembly of the LHCII trimer and its subsequent degradation, while general dephosphorylation of PSII proteins may be involved in repair cycle of PSII proteins and stress-response-signaling.

1. Introduction

The phosphorylation of several proteins in thylakoid photosynthetic membranes of plant chloroplasts has been studied for more than 30 years. Many phosphorylated proteins have been identified, such as D1, D2, CP43, PsbH, TMP14, PsaD, and some polypeptides belonging to the light-harvesting complex (LHCII), including CP29 [1,2]. Reversible phosphorylation of these membrane proteins was found to be light- and redox-regulated in chloroplasts or thylakoids isolated from different species [2,3]. Application of biochemical techniques detecting *in vivo* phosphorylated proteins has also revealed differential environment-dependent changes in phosphorylation state of the photosynthetic proteins, especially in stressful conditions. Enhanced phosphorylation of PSII polypeptides has been found in plants and green algae subjected to high light stress, long-term drought stress, nutrition deficiency, or low temperature [2]. On the contrary, extremely fast dephosphorylation of the D1, D2 and CP43 proteins was identified in plant leaves as an immediate response to abrupt elevation of temperature [4]. Specific phosphorylation of CP29, the minor light-harvesting protein of PSII, has been discovered in plants subjected to cold stress [5] and combined high light and cold treatment [6]. Although several protein kinases involved in phosphorylation of thylakoid proteins have been found [7–9], and the difference of phosphorylation conditions of different PSII proteins also has been documented [10], the molecular mechanisms of these regulatory events remain largely unknown. Furthermore, there are few reports about phosphorylation levels of PSII proteins under water or osmotic stress. We currently know that long-term drought stress increases D1 phosphorylation in *Pisum sativum* [11] and osmotic stress may decrease LHCII phosphorylation in higher plants [12,13]. But the systematic researches are largely short.

Effects of water stress on PSII have also been well studied. Previous reports showed that the two photosystems, PSII and PSI, particularly PSII, were affected by water stress and this led to lowered electron transport through them [14–17]. The steady-state-levels of PSII proteins D1, D2 and LHCII and the corresponding mRNA levels of genes *psbA*, *psbD* and *cab* also decrease dramatically under osmotic stress, which can be attributed to decreased transcription and translation rates and accelerated degradation of proteins and mRNAs [14,18,19].

Abbreviations: Chl, chlorophyll; DM, n-dodecyl-β-D-maltoside; LHCII, light-harvesting complex II; OG, n-octyl-β-D-glucopyranoside, PSII, photosystem II; q_P, Photochemical quenching

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It is well known that PSII protein turnover is closely related to their phosphorylation state. In this paper, we report dephosphorylation of PSII proteins and phosphorylation of CP29 in barley seedlings in response to water stress. Lateral migration from granum membranes to stroma-exposed lamellae was only found to CP29, and the other PSII proteins may not migrate and be degraded directly *in situ*.

2. Materials and methods

2.1. Plant materials and stress treatment

Barley (*Hordeum vulgare* L.) were grown in sand and cultured with 1/2 Hoagland solution at 25 ± 1 °C under a 12-h photoperiod and the photosynthetic photon flux of 100 photons μmol $m^{-2}s^{-1}$. When grown to the third-leaf stage (14 d after germination), seeding were removed from the sand, washed with tap water and dried briefly with paper towels to remove surface water. Water stress was initiated by submerging the roots into nonpenetrating PEG solution (aerated with air) [20], with 20 mM NaF, with an osmotic potential of −0.5 MPa in beakers. Control plants were grown in 1/2 Hoagland solution and all samples were treated for 24, 48 and 72 h under the above conditions, then the sample stress stressed for 72 h was rewatered in Hoagland solution without PEG for 24, 48 and 72 h. The results of stress were characterized by the relative water content (RWC).

2.2. Isolation and subfractionation of thylakoid membranes

Thylakoid membranes were isolated as described by Suorsa et al. [21] with 20 mM NaF. Then The thylakoid membranes were mechanically broken by shaking (without sonication) and then purified by aqueous two phase system to isolate the grana and stroma lamellae fractions, and the grana fraction was further purified to isolate the grana core and the grana margins fractions by sonication, according to Jansson et al. [22] and Danielsson et al. [23].

2.3. SDS-PAGE, protein staining and western blot analysis

According to the method of Tikkanen et al. [24], isolated thylakoids were solubilised in the presence of 6 M urea, and the polypeptides were separated by SDS-PAGE using 15% (w/v) acrylamide gels with 6 M urea. Pro-Q Diamond (Molecular Probes) Phosphoprotein gel stain was used according to manufacturer's instructions to analyze the phosphorylation level of thylakoid proteins. For negative control, samples were incubated with alkaline phosphatase (Sigma) in a buffer containing 0.1 M glycine, pH 10.4, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$ for 30 min at room temperature.

Quantities of proteins were measured according to standard Bradford method [14]. For Western blotting, the proteins were electron-transferred onto a nitrocellulose film according to Yuan et al. [14]. Than antisera to the D1 (provided by Dr. Eva-Mari Aro), LHCII b1 and b4 (purchased from AgriSera Comp., Stockholm, Sweden), FtsH (gifts from Prof. Shigemi Seo and Prof. Zach Adam), DegP (gifts from Prof. Zach Adam), SppA (purchased from AgriSera Comp., Stockholm, Sweden), and TLP40 (gift from Prof. R. Herrmann) were applied. The signals were revealed by using secondary antibodies of alkaline phosphatase goat anti-rabbit IgG. The intensity of the signals of Western blotting was analyzed densitometrically by a thin-layer scanner.

2.4. Native green-gel electrophoresis

The native green-gel electrophoresis of thylakoid membranes, PSII core particles, and LHCII were determined according to the procedure of Lin et al. [25] and Tang et al. [26] using a tube gel with 12% resolving gel and 5% stacking gel. Thylakoids were washed twice in ice-cold 2 mM Tris–maleate (pH 7.0) and then solubilized for 30 min on ice in a solution consisting of 0.45% n-octyl-β-D-glucopyranoside (OG), 0.45% n-dodecyl-β-D-maltoside (DM), 0.1% SDS, 10% glycerol and 2 mM Tris–maleate (pH 7.0). Unsolubilized material was removed by centrifugation at 20,000 ×*g* for 10 min and the supernatant was loaded onto the gels (60 μg Chl/tube).

2.5. Assay of protein phosphatase activity with phosphopeptide substrates

^{32}P-labeled phosphopeptides were prepared from thylakoids phosphorylated with [γ-^{32}P]ATP in the light, and cleaved from membranes with trypsin, as described in Vener et al. [27]. To assay protein phosphatase activity, the phosphopeptides (cleaved and the remains of membrane-bound phosphopeptides) were resuspended in a buffer containing 50 mM Tricine, pH 7.8, 100 mM sorbitol, and 5 mM $MgCl_2$. Then 5 μL of the phosphopeptides (about 20 μM) and 5 μL of thylakoids (1 mg chlorophyll mL^{-1}, without NaF) were mixed and incubated for 3 to 20 min at 25 °C. The amount of ^{32}P-label remaining in the phosphopeptides and the released labeled ^{32}Pi were determined by the acid molybdate extraction of ^{32}Pi as described before [27].

2.6. Dephosphorylation of thylakoid protein in vivo

According to Rokka et al. [4] with some modifications, barley plants were illuminated under a PFD of 1000 μmol photons $m^{-2}s^{-1}$ at 25 °C for 60 min to phosphorylate PSII reaction center proteins. To induce maximal LHCII phosphorylation, barley leaves were illuminated at low light (a PFD of 80 μmol photons $m^{-2}s^{-1}$) for 60 min. After light treatment the barley leaves were transferred to darkness and incubated at 25 °C for up to 120 min to dephosphorylation gradually. Samples for thylakoid isolation were taken during the time course of incubation, frozen in liquid nitrogen, and stored at −80 °C.

2.7. Gas exchange and fluorescence measurements at room temperature

Gas exchange analysis was made using an open system (TPS-1, PP system, UK). Chlorophyll fluorescence quenching analysis was carried out at room temperature (25 °C) with a portable fluorometer (PAM-2100, Walz, Germany) as determined by Yuan et al. [15].

2.8. Low temperature (77 K) fluorescence emission spectra

Barley leaves from different treatments were immediately frozen in liquid nitrogen. Leaves were then grinded in buffer containing 50 mM Hepes–KOH, pH 7.5, 100 mM sorbitol, 10 mM $MgCl_2$, and 10 mM NaF. After filtration, the samples were diluted to a chl concentration of 10 μg ml and the 77 K fluorescence emission spectra were immediately recorded with a diode array spectrophotometer (S2000; Ocean Optics, Dunedin, FL, USA) equipped with a reflectance probe. Fluorescence was excited with white light below 500 nm, defined by using LS500S and LS700S filters (Corion Corp., Holliston, MA, USA). The emission between 660 and 785 nm was recorded.

2.9. Electron microscopy

Samples from differently treated leaves were fixed overnight at 4 °C in 3% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 6.9) and then processed for electron microscopy according as described [25]. Ultrathin sections, cut with an ultramicrotome (Ultracut, Reichert-Jung), were observed with a transmission electron microscope (TEM 300, Itachi) operating at 75 kV.

2.10. Statistical analysis

Means of 5 triplicates were measured. Student's *t* test was used for comparison between PEG and PEG+NaF. A difference was considered to be statistically significant when $p<0.05$.

3. Results

3.1. Inhibition of dephosphorylation aggravated water stress and hampered photosystem recovery

Chlorophyll fluorescence quenching analysis has been proven a non-invasive, powerful and reliable method to assess the changes of the function of PS II in the steady state of photosynthesis in response to different environmental stresses [15,17,28]. Supplementary Fig. S1 shows that F_v/F_m, F'_v/F'_m, Φ_{PSII} and q_P declined gradually in all plants as the water stress developed, which were consistent with the results of Lu et al. [28]. Sole NaF pretreatment did not apparently affect PSII photochemical activities (data not shown), but NaF+PEG treatment decreased these fluorescence parameters more quickly. NaF also hampered recovery of PSII during rewatering, and it could not recover to control levels.

Low temperature fluorescence emission reflected a similar trend that PSII was more sensitive to water stress than PSI, and NaF hampered PSII, especially its recovery during rewatering (Supplementary Fig. S2). It is interesting that low temperature fluorescence emission of PSI also decreased dramatically in NaF-treated seedlings, and could not be recovered after rewatering. This fact may point that dephosphorylation plays an important role in adaption of PSI to water stress. PsaD and TMP14 are the only PSI protein that can be phosphorylated [1,29], and may be important to stress adaption [30]. Although NaF may be toxic to plants and have some side effects, all above parameters still are consistent with the idea that dephosphorylation is an essential step of PSII protein turnover and resistance to water stress.

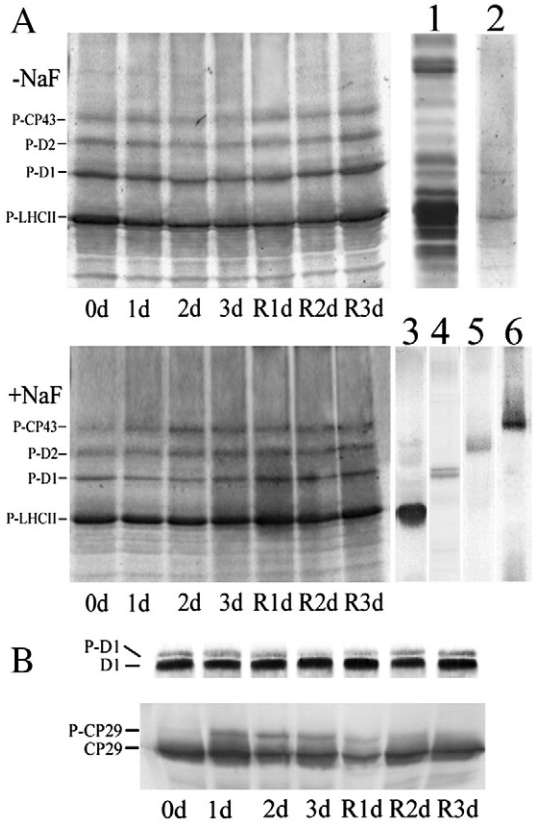

Fig. 1. Steady-state-levels of phosphorylated proteins in thylakoid membranes under water stress and rewatering. 0d represents control seedlings; 1–3d represents water stress for 1–3 d; R1–3d represents rewatering for 1–3 d after 3 d of water stress. Barley seedlings were randomly divided into two groups for the following treatment. One group was submerged to nonpenetrating PEG solution (−NaF). The other group was submerged to nonpenetrating PEG solution with 20 mM NaF (+NaF). (A) Phosphorylation of thylakoid proteins was determined by Pro-Q® Diamond Phosphoprotein Gel Stain. (B) Phosphorylation of thylakoid proteins was determined by immunoblotting with specific antibodies. For the comparison of one sample with another, gel lanes were loaded on an equal thylakoid protein basis. Line 1 shows the SYPRO® Ruby Protein Gel Stain of total thylakoid proteins. Line 2 shows the Pro-Q® Diamond Phosphoprotein Gel Stain of phosphatase-treated thylakoid proteins. The bands of phosphorylated LHCII, D1, D2, CP43 were confirmed by specific antibodies (lines 3–6 respectively).

3.2. Phosphorylated PSII proteins decreased but phosphorylation of CP29 increased under water stress

Pro-Q® Diamond Phosphoprotein Gel Stain showed steady-state-levels of phosphorylated proteins in thylakoid membranes under water stress (Fig. 1A). Each loading was derived on an equal thylakoid protein basis, and the gels were either stained for protein analysis or transferred for Western blotting (Fig. 1B). All visible phosphorylated thylakoid proteins remarkable decreased under water stress, and could be reversed by rewatering, including D1, D2, CP43 and LHCII. If the seedlings were pre-treated with NaF, these changes were eliminated. Total LHCII proteins decreased a little during water stress [14], also reflected in the NaF-treated sample (Fig. 1A). Contrastively, phosphorylated LHCII largely decreased in water-stressed seedlings without NaF treatment (Fig. 1A), suggesting a combination of dephosphorylation of a constant pool of LHCII, and degradation of LHCII itself. Immunoblotting with specific antibodies confirmed the decline of phospho-D1 (Fig. 1B). During the first 24 h, phosphorylation of CP29 was quickly induced. And after rewatering, the bands of phospho-CP29 became dramatically weaker. CP29 phosphorylation changed inversely to other PSII proteins.

3.3. Water-stress-stimulated thylakoid protein dephosphorylation in vivo

In vivo experiments were performed to investigate the physiological relevance of the water-stress-induced specific dephosphorylation of PSII. Only very slow dephosphorylation of the D1 and CP43 proteins occurred in the leaf discs during a 120 min incubation in darkness at 25 °C as judged by analysis with Pro-Q® Diamond Phosphoprotein Gel Stain or high-resolution gel separation [31] for phosphorylated and non-phosphorylated D1 protein (Fig. 2A–C). The relative amount of phospho-LHCII formed during the incubation of control leaves at low light almost did not change upon transfer of leaves to darkness. On the contrary, water stress accelerated dephosphorylation of LHCII proteins (Fig. 2D). Although phospho-CP29 was induced by water stress, its dephosphorylation accelerated at the same time (detected by CP29 specific antibody, Fig. 2E). Its dephosphorylation pattern is similar to other LHCIIs.

Table 1 shows the dephosphorylation ratios of CP43, D1/D2, LHCII, and the 29-kD protein. Dephosphorylation behavior of all these proteins is similar. In the untreated condition (CK), all PSII proteins dephosphorylated very slowly (half-times were more than 120 min). Water stress increased the dephosphorylation rates of both PSII reaction center proteins and light-harvesting proteins. Only one-day water stress could cause significant increase of dephosphorylation of PSII proteins. Under severe water stress (PEG for 72 h), dephosphorylation rates of all these proteins decreased to about 47–58 min. Rewatering would gradually decelerate dephosphorylation of PSII proteins.

3.4. Both membrane-bound and extrinsic phosphatases were activated during water stress

Thylakoids contain two kinds of protein phosphatases. Membrane-bound phosphatase has a higher specificity to PSII core proteins rather than LHCII [4,27], while extrinsic thylakoid phosphatases have been shown to dephosphorylate mostly LHCII, but not PSII proteins [4,32]. To determine whether the thylakoid protein phosphatase is highly activated by water stress, the activity of phosphatases from intact

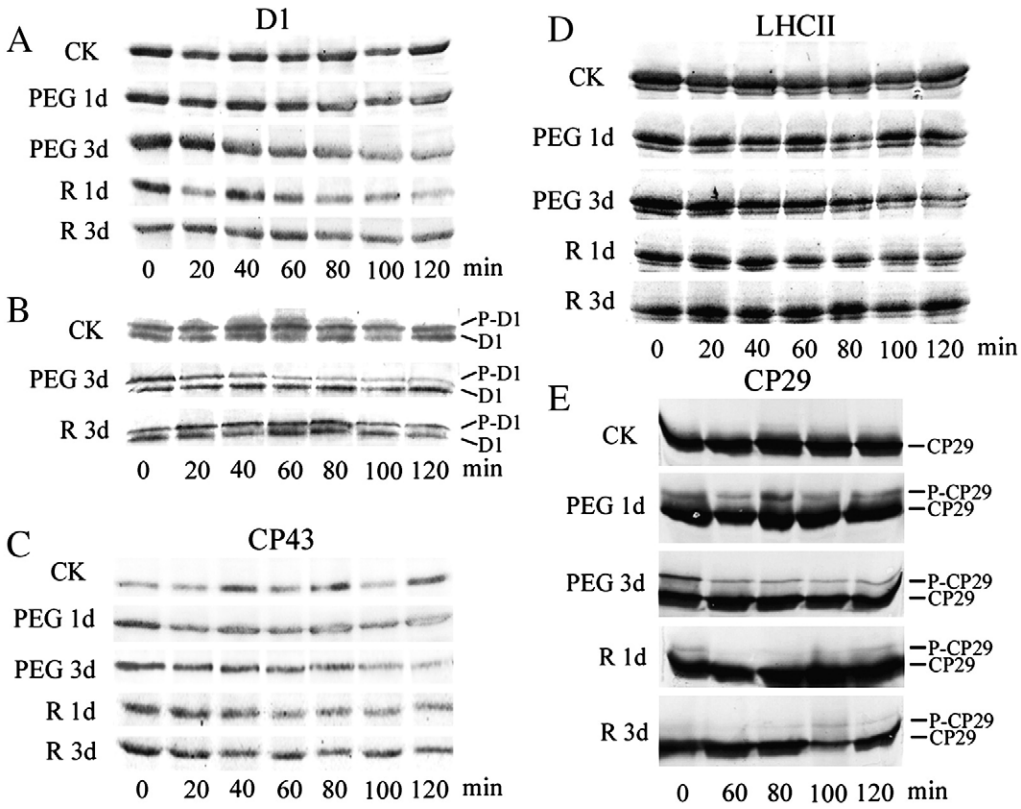

Fig. 2. Dephosphorylation of thylakoid proteins *in vivo* under water stress and rewatering. Barley seedlings were illuminated 60 min at 25 °C and then transferred to darkness and incubated at 25 °C. Dephosphorylation was terminated at the indicated time points by freezing the leaves in liquid nitrogen. Thylakoid membranes were isolated and the extent of protein phosphorylation was determined using a Pro-Q® Diamond Phosphoprotein Gel Stain (A, C and D) or a D1-specific antibody (B) or a CP29-specific antibody (E). In the latter case the upper band of the D1/CP29 doublet represents the phosphorylated form of the protein, indicated by P-D1 and P-CP29. Before conducting the dephosphorylation experiments different light intensities were used for induction of higher *in vivo* phosphorylation levels of either PSII core proteins or LHCII. The leaves were illuminated under a PFD 1000 μmol photons m^{-2} s^{-1} for more effective phosphorylation of PSII proteins (A, B and C) or under a PFD 80 μmol photons m^{-2} s^{-1} for induction of LHCII phosphorylation (D and E). CK represents control seedlings; PEG 1–3d represents water stress for 1–3 d; R1–3d represents rewatering for 1–3 d after 3 d of water stress.

thylakoids was assayed for different treatments using truncated thylakoid phosphopeptides as substrates. These ^{32}P-labeled peptides cleaved from membranes by trypsin are primarily PSII core phosphopeptides of D1, D2 and CP43, and some short and nonacetylated phosphopeptides of LHCII, while the ^{32}P-labeled remains mostly are phosphopeptides of LHCII [27]. Dephosphorylation of PSII core phosphopeptides increased as the water stress developed, and decreased after rewatering (Fig. 3A). Dephosphorylation of phosphopeptides of LHCII changed with a similar pattern (Fig. 3B). Therefore, the activities of both membrane-bound and extrinsic protein phosphatases may be enhanced under water stress.

3.5. Redistribution of PSII in thylakoids supports no thylakoid destacking happened

To gain additional insight on the effect of water stress on the distribution of PSII, thylakoids from stressed plants were fractionated into granum-, margin-, and stroma-enriched fractions. Then the protein content of each fraction was determined. Grana region is the largest part of the thylakoid (about 42%), and it increased to 56% after 3d of water stress. Correspondingly, margin- and stroma-enriched fractions decreased relatively (Supplementary Fig. S3). We interpret this phenomenon as the consequence of degradation of lateral membranes faster. Thylakoid membranes are highly appressed and PSII is concentrated in the stacked grana regions, which may make it poorly accessible to either a soluble phosphatase or the catalytic domain of a membrane enzyme [33]. Therefore the stroma-enriched fraction is more easily degraded than the grana margins than the grana core. Although total thylakoid proteins decreased by about half after water stress [14,18], the unstacked membranes degraded much faster. Consequently, the proportion of grana region increased relatively. Electron micrographs of water-stressed barley leaves showed no destacking of the membrane (Fig. 4). Both the destacked thylakoids and the appressed regions decreased largely after water stress. The grana numbers decreased from 32.1 ± 2.4 grana per plastid to 14.7 ± 1.6 grana per plastid after 3 d of water stress, but the grana size were almost not changed, ranging from 3.4 ± 0.8 μm to 3.1 ± 0.7 μm (not significantly different). Neither swelling of thylakoid membranes, nor partial relaxation of the granum structure could be seen. This finding contradicts with what is known about destacking of grana after irradiation with ultraviolet B. In the latter case, upon ultraviolet stress, dismantling of grana occurs as the consequence of the dismantling of PSII core proteins that are involved in membrane stacking [34].

Table 1
Water stress dependence of the dephosphorylation rates for the major phosphoproteins in isolated thylakoids.

Phosphoprotein	$t_{1/2}$ (min)				
	CK	PEG 1d	PEG 3d	Rewater 1d	Rewater 3d
CP43	>120	89 ± 25	58 ± 15	108 ± 23	>120
D1/D2	>120	120 ± 27	47 ± 12	82 ± 19	>120
LHCII	>120	116 ± 31	52 ± 18	63 ± 22	>120
CP29	>120	>120	54 ± 21	89 ± 18	>120

The data are presented as the half-times (minutes). The half-times were calculated from the first-order rate fitting of the dephosphorylation versus time curves obtained from three experiments at each treatment.

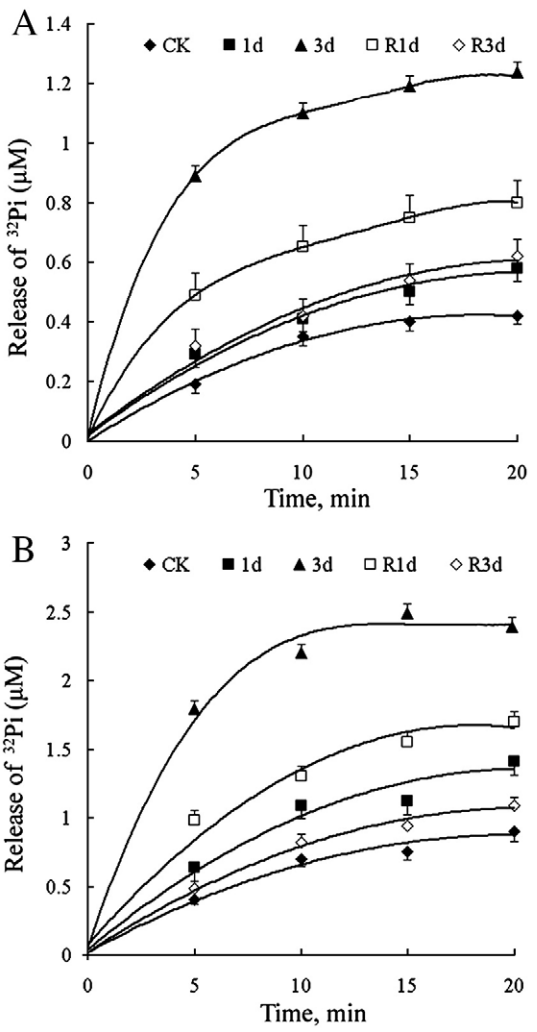

Fig. 3. Changes of phosphatase activity of intact thylakoid membranes under water stress and rewatering. The ^{32}P-labeled phosphopeptides were obtained from radioactively labeled thylakoid membranes by trypsin treatment. Then the ^{32}P-labeled peptides cleaved from membranes (A) and the left ^{32}P-labeled peptides binding with membranes (B) were used as substrates and incubated with intact thylakoids from different treatments for phosphatase assays. The phosphatase activity is indicated by the release of ^{32}Pi. CK represents control seedlings; 1–3d represents water stress for 1–3 d; R1–3d represents rewatering for 1–3 d after 3 d of water stress. Bars represent standard deviations of 3 independent replicates.

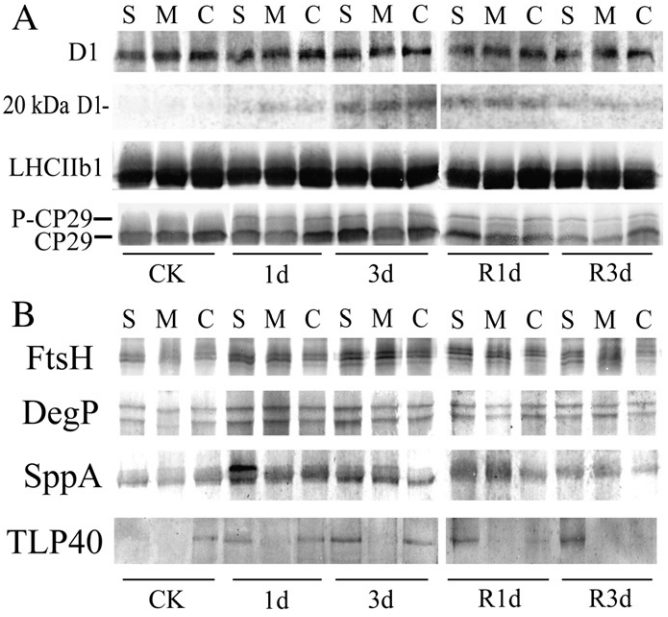

Fig. 5. Distribution of PSII proteins (A), proteases and TLP40 (B) on thylakoid membranes under water stress and rewatering. Thylakoids from different treatments were subfractionated into grana core (C), grana margins (M), and stroma-exposed (S) regions. D1, LHCIIb1, CP29, FtsH, DegP, SppA and TLP40 were detected using specific corresponding antibodies. D1 breakdown fragments are indicated by 20 kDa. Gel lanes were loaded on an equal protein basis (20 μg). CK represents control seedlings; 1–3d represents water stress for 1–3 d; R1–3d represents rewatering for 1–3 d after 3 d of water stress.

3.6. Lateral migration only happened to CP29 but not other phosphorylated PSII proteins

A previous study showed that heat shock accelerates dephosphorylation of PSII, which could be attributed to partial temperature-induced dissociation of LHCII from PSII, partial unstacking of thylakoids, and lateral migration of PSII to the stroma-exposed thylakoid regions [4]. Water stress also accelerated dephosphorylation of PSII, but the other subsequent transformations as mentioned above did not occur during water stress. Water stress did not cause a lateral migration of PSII from grana as judged from the distribution of PSII proteins between stacked and unstacked thylakoid fractions (D1 and LHCIIb1 in Fig. 5A; data of D2, CP43 and LHCIIb2–6 are not shown). The breakdown fragments of D1 increased under water stress. CP29

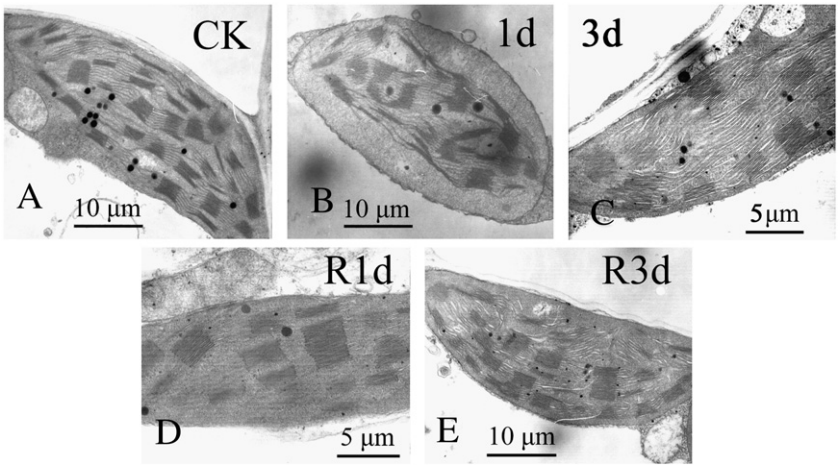

Fig. 4. Electron micrographs of barley seedlings treated with PEG for 0 d (A), 1 d (B), and 3 d (C), and then rewatering for 1d (D) and 3d (E). CK represents control seedlings; 1–3d represents water stress for 1–3 d; R1–3d represents rewatering for 1–3 d after 3 d of water stress.

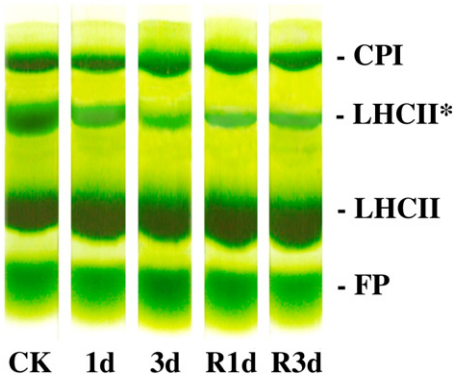

Fig. 6. Chlorophyll–protein complexes separated by mild denaturing electrophoresis from barley seedlings of different treatments. CP1 represents photosystem I core complex; LHC II* represents LHC II trimers; LHC II represents LHC II monomers; FP represents free pigments. CK represents control seedlings; 1–3d represents water stress for 1–3 d; R1–3d represents rewatering for 1–3 d after 3 d of water stress.

presented an opposite change that it laterally migrated from stacked fractions to stroma-exposed fractions after 3d water stress (Fig. 5A). Rewatering reversed the above regulation.

Fig. 6 shows the native green-gel electrophoresis of barley thylakoids. The results showed that LHCII trimers disassembled to oligomers or monomers and dissociated from PS II complex, which could not be recovered by rewatering. However, the detached LHC II did not migrate from the stacked membranes to the unstacked membrane regions or bind to PS I complex (Fig. 5A). It seems that LHCII (except CP29) disassembled and degraded directly *in situ*. Reassembly may be time-consuming, therefore the LHCII trimers could not be increased immediately after rewatering.

3.7. Activation of plastid proteases and release of TLP40 from the thylakoid membrane

In plants, the proteolytic machinery of chloroplasts consists primarily of some families of ATP-dependent proteases, Clp, FtsH, SppA et al., and one family of ATP-independent proteases, DegP. Most of them are involved in degradation of PSII core proteins [35–37]. And FtsH6 is a general LHC II protease [38]. We used polyclonal antibodies of FtsH, SppA and DegP to detect expression and distribution of thylakoid proteases under water stress. All three kinds of proteases localize to every part of thylakoid, and significantly increased after water stress, which could be reversed by rewatering (Fig. 5B).

The peptidyl–prolyl cis–trans isomerase TLP40 can regulate the activity of the protein phosphatase within the thylakoid membrane [27]. It was proposed that binding of TLP40 to the intrinsic phosphatase depresses the dephosphorylation activity, whereas activation of the phosphatase occurs upon release of TLP40 into the lumen [27]. At control, most TLP40 was bound to the membrane, and almost no protein was soluble in the lumen (Fig. 5B). After water stress, a much greater fraction of the protein was released from the membrane, which could not be apparently reversed by 3d of Rewatering. After subfractionation of thylakoid membranes, luminal proteins and stromal proteins are mixed together. Therefore, released TLP40 presented in stroma-exposed thylakoid region.

4. Discussion

4.1. Reasons and significance of PSII protein dephosphorylation under water stress

In this paper we describe decreases of steady-state-levels of phosphorylated proteins in thylakoid membranes under water stress. Two hypotheses may explain these decreases. One is the redox change in the plastid induced by water stress. The redox state of PSII ($1-q_P$) can be monitored by measuring the photochemical quenching of chlorophyll fluorescence (q_P) [6,10]. From Fig. S1 we know that q_P declined gradually after water stress, thus reduction of the plastoquinone pool decreased, and thylakoid protein kinases should be deactivated [6,10]. Another reason is the accelerated dephosphorylation.

The general dephosphorylation of the PSII proteins under water stress conditions should play an important regulatory role, as dephosphorylation of D1 and D2 proteins do in the repair cycle of PSII in response to light stress [3,31]. Only after dephosphorylation can the D1 protein be proteolytically degraded [31,39]. Thus rapid dephosphorylation of the PSII reaction center proteins appears to be a regulatory reaction to water stress in plant photosynthetic membranes. Our previous study showed that degradation of D1/D2 increases under water stress [14], which also supported our conclusion. But the physiological role of dephosphorylation of LHCII is not clear. It is known that non-phosphorylated LHCII should associate with PSII and not migrate laterally [2]. Therefore, accelerated dephosphorylation of LHCII may keep the LHCIIs *in situ* for subsequent degradation.

4.2. PSII proteins degraded in situ under water stress

Fractionation of thylakoid membranes into grana, margins, and stroma-exposed lamellae indicates that there is no significant migration of major PSII proteins between the different compartments of the thylakoid membrane (Fig. 5). Inspection of our electron micrographs of chloroplasts from water-stressed plants indicates that no destacking and no partial relaxation of the granum occurred (Fig. 4). Thus two possible processes can be deduced. One possibility is that all PSII proteins degrade *in situ*, while the proteins in stroma-exposed fractions degrade faster. Another case is that damaged PSII proteins may migrate to unstacked thylakoid fractions, but degrade very fast then, therefore cannot be detected. However, we consider the first assumption most plausible. In fact, water-stress-stimulated dephosphorylation of LHCII proteins is a condition expected to retain LHCII proteins in the granum membranes rather than to promote their migration into stroma-exposed lamellae [2,34]. Moreover, the major thylakoid proteases and D1 breakdown fragments distribute in every part of barley thylakoids (including granum), and the granum proteases can also be enhanced under water stress (Fig. 5). The granum proteases should indeed hydrolyze PS proteins *in situ*.

4.3. Reasons and significance of CP29 phosphorylation under water stress

The phosphorylation and migration pattern of CP29 contradicts to all other thylakoid phosphoproteins during water stress. CP29, like other LHCIIs, is under the control of the STN7 kinase [24]. However, its phosphorylation is largely different from other LHCIIs or D1/D2 and CP43. Phosphorylation of D1, D2 and CP43 was directly dependent on the reduction state of the plastoquinone pool. Complete phosphorylation of Lhcb1 and 2 proteins, on the contrary, required only modest reduction of the plastoquinone pool, and was subject to inhibition upon increase in the thiol redox state of the stroma. While, phosphorylation of CP29 occurred upon strong reduction of the plastoquinone pool, and was further enhanced by low temperatures. *In vitro* studies further demonstrated that CP29 phosphorylation is independent of the redox state of both the cytochrome b_6/f complex and the thiol compounds [10]. In this paper, we found rapid phosphorylation of CP29, even within first 24 h of water stress. Photochemical quenching (q_P) was not significantly changed at that time (Supplementary Fig. S1). Therefore, water-stress-induced CP29 phosphorylation is independent of redox state of plastoquinone pool. It should be boosted through an unknown mechanism, which requires further studies.

In *Arabidopsis* [24] and *Chlamydomonas* [40], it was suggested that CP29 phosphorylation might be important for the mechanism of state transitions. It is conceivable that CP29 phosphorylation occurs only in those PSII–LHCII supercomplexes that participate in state transitions, thereby modulating the forces in the PSII–LHCII supercomplexes in the grana and resulting in the migration of a portion of LHCII from the PSII core to the stroma thylakoids in order to serve the light-harvesting function for PSI [24]. Kargul et al. [40] even find that phosphorylated CP29 can bind to photosystem I of *Chlamydomonas reinhardtii*, and suggest that CP29 is a connecting protein between PS core and movable LHCII [41,42]. Here in barley, water-stress-induced CP29 phosphorylation may facilitate disassociation of LHCII from PSII complex, disassembly of LHCII trimer and its subsequent degradation. Previous studies also implied that CP29 plays important roles in the assembly of PSII into the supercomplex and the macroorganization of supercomplexes in the thylakoid membrane [42]. These structural features of PSII are required to maintain its high quantum efficiency. Besides, phosphorylation of CP29 may induce a conformational change that modifying the chlorophyll organization, and lead to non-radiative energy dissipation [5,41,43]. Actually, nonphotochemical quenching (NPQ) increased a little during water stress [15,28].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.04.012.

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